Plant Physiol. (1991) 96, 843–847
0032-0889/91/96/0843/05/$01.00/0

Received for publication January 10, 1991
Accepted March 27, 1991

Effect of Fe-Catalyzed Photooxidation of EDTA on Root Growth in Plant Culture Media

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ABSTRACT

Light from fluorescent lamps can induce formaldehyde production and iron deficiency in plant nutrient culture medium. Formaldehyde is produced from EDTA when it is oxidized by the photochemical reduction of ferric iron and it can accumulate to inhibitory levels. The medium becomes iron deficient because iron becomes unchelated and forms an unavailable precipitate as EDTA is oxidized. The combination of light-induced formaldehyde production and loss of available iron reduces the ability of the culture media to support growth of *Arabidopsis thaliana* roots. Removing ultraviolet and blue wavelengths with a yellow acrylic filter is a simple and effective means of preventing Fe-catalyzed photooxidation of EDTA in plant culture media.

Recent investigations showed that IAA degradation in plant tissue culture media is catalyzed by the photodynamic activity of Fe-EDTA (3, 14). Light-induced IAA degradation can result in reduced growth for tissues that require auxin for cell proliferation (3, 14). Light-induced degradation of hormone-free medium has also been reported recently (14). The growth of *Arabidopsis* roots was reduced when they were grown on hormone-free nutrient medium that had been previously exposed to light. These data suggest that some unidentified inhibitory material was photochemically produced and/or necessary growth factors were made unavailable (14). We have investigated the photochemistry involved in these light-induced changes in medium composition because they could be an important source of variability in the growth of cultured tissues.

Because Fe-EDTA is the major light-absorbing component in plant culture media (14), it seemed to be the most likely source of the light-induced growth reduction observed with *Arabidopsis* roots. EDTA is an effective electron donor for photoreducing flavins (4, 5, 6, 11), and photoreduction of Fe^{3+} to Fe^{2+} by UV and blue radiation is enhanced by certain di- and tricarboxylic acids which can serve as electron donors (1). Moreover, the aerobic reaction products from flavin-catalyzed oxidation of EDTA in the light are glyoxylic acid, carbon dioxide, an amine residue, and formaldehyde, which originates from the carbon adjacent to the carboxyl group (4, 6). Thus, if EDTA is an electron donor to Fe^{3+}, the degradation products of EDTA may be at least partially responsible for the reduced growth observed in illuminated medium (14). For example, the accumulation of formaldehyde would be toxic to biological systems. In addition, once the EDTA is degraded the iron will no longer be chelated and available to the plant tissues. Therefore, we investigated the possibility that formaldehyde formation and loss of available iron are responsible for the reduction of *Arabidopsis* root growth observed in light-exposed culture media.

MATERIALS AND METHODS

Plant Material

Seeds of *Arabidopsis thaliana* ecotype Columbia were surface-sterilized for 15 min in 1.5% sodium hypochlorite. After rinsing with sterile water, the seeds were germinated and grown for 3 d at 22 to 24°C under continuous illumination (65 μmol m^{-2} s^{-1} PPFD) on Murashige and Skoog nutrients (12) containing 0.8% agar and 3% sucrose in polystyrene Petri dishes.

Root Elongation Assay

Three-day-old *Arabidopsis* seedlings were transferred to Petri dishes containing growth medium that had been exposed to the indicated light regimens. For agar-solidified medium, the root tip positions were marked and the dishes were oriented vertically so the roots grew on the surface. The increase in root length was measured after 2 d in darkness. For liquid medium, the dishes were incubated on a rotary shaker at 30 rpm. Root lengths were measured after 2 d and the growth increase was calculated by subtracting the initial mean root length measured from a random sample of 20 plants harvested at the time of transfer. Under these conditions, root growth in fresh or dark-incubated medium varied from 10 to 14 mm between experiments, but within an experiment the SE was close to 5%. Each experiment was repeated at least three times.

Light Conditions

Light was supplied from cool white fluorescent lamps (GE F96T12-CW). The PPFD at the surface of the Petri dishes was measured with an LI-185B quantum photometer (LI-COR, Inc., Lincoln, NE). Light levels were varied by changing the distance between the Petri dishes and the source. The polystyrene Petri dishes used for all experiments cut off light below 290 nm, which corresponds to our standard unfiltered light conditions. Acrylic yellow-2208 (3.18 mm thick, Polycast Technology Corp., Stanford, CN) was used to block wavelengths below 454 nm (14).

Formaldehyde and Glyoxylic Acid Determination

Formaldehyde and glyoxylic acid were quantified by HPLC of their 2,4-DNPH[1] derivatives (8, 10). Derivatization followed a modification of the procedure of Graven *et al.* (7). To 1 mL aliquots of the medium, 30 μL of 12.6 mM 2,4-dinitrophenylhydrazine in 6 N HCl were added. The mixture was stirred and allowed to stand for at least 5 min. The derivatives were stable for at least 5 h.

Derivatized samples (20 μL) were chromatographed on a 4.6 mm i.d. × 250 mm Hibar Ec cartridge containing Merck Lichrosorb RP-18 10 μM C18 reverse phase packing (Alltech Associates, Deerfield, IL) protected by a guard column containing the same packing. The mobile phase was 60% acetonitrile/water delivered by an Applied Biosystems Spectraflow model 430 low pressure gradient former and an Applied Biosystems Spectraflow model 400 high pressure pump at a flow rate of 1.5 mL min^{-1}. Detection was at 380 nm with an Applied Biosystems Spectraflow 783 absorbance detector coupled to a Spectra-Physics SP4290 integrator. Calibration was achieved using standard solutions of formaldehyde and glyoxylic acid that had been subjected to the same derivatization procedure.

Purified formaldehyde-2,4-DNPH was prepared by adding 2 mL of the 2,4-dinitrophenylhydrazine reagent to 50 mL of a 100 mM solution of formaldehyde. Crystalline formaldehyde-2,4-DNPH was removed by filtration and recrystallized from ethanol. Structural confirmation of formaldehyde-2,4-DNPH was accomplished by GC-MS for both the authentic material and representative media samples at the Ohio State University Chemical Instrument Center, by use of an HP 5970B GC/MSD system. For GC-MS, the derivatized media samples were extracted into dichloromethane prior to analysis (7).

Determination of Soluble Chelated Iron

The blue color formed by the reaction of ferrocyanide with ferric iron was used to measure the amount of soluble chelated iron in solutions of Fe-EDTA (13). Samples of 1 mM Fe-EDTA that had been exposed to various light treatments were centrifuged at 2000*g* to remove insoluble iron oxides. The supernatant was acidified to pH 1.8 with HCl and 2 mL were mixed with 2 mL of 1 mM potassium ferrocyanide. The color was allowed to develop for 20 min and the absorbance at 766 nm was measured. The response was linear between 0.01 and 2.0 mM with Fe-EDTA solutions.

RESULTS AND DISCUSSION

Prior incubation of hormone-free Murashige and Skoog medium (12) under fluorescent lights resulted in reduced root growth of *Arabidopsis* seedlings that were placed on the preilluminated medium and allowed to grow in the dark (14). The light-induced changes in Murashige and Skoog medium that causes the reduction of root growth is light intensity-dependent (Fig. 1). At the highest light level tested (200 μmol m^{-2} m^{-1}), root growth was totally inhibited after the medium had been incubated for 20 d. From these experiments, two types of light-induced change seemed possible: the production of toxic material and/or the loss of growth-promoting components of the medium.

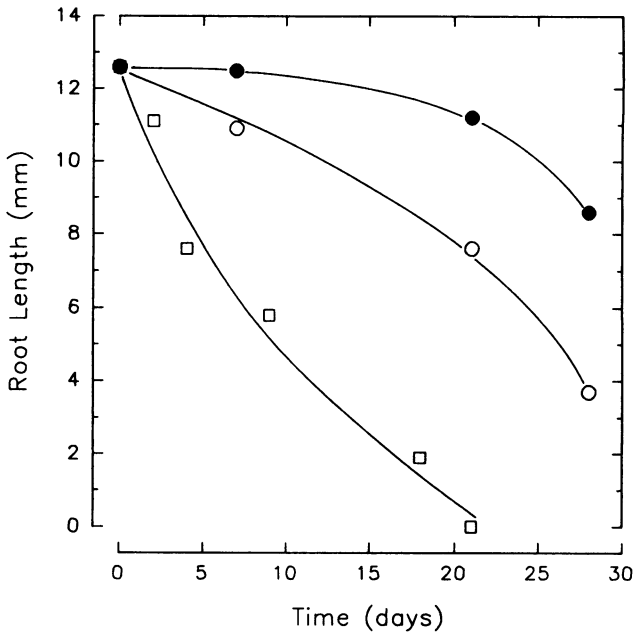

Figure 1. Effect of prior exposure of culture medium to different light intensities on the growth of *Arabidopsis* roots. Medium was exposed to 25 μmol m^{-2} s^{-1} (●), 65 μmol m^{-2} s^{-1} (○), and 200 μmol m^{-2} s^{-1} (□) of white light at the surface of the Petri dishes. Three-day-old seedlings were then placed in the preincubated media and their root growth was measured after incubation in the dark for 2 d. This experiment was conducted with liquid medium, but similar results were obtained with agar-solidified medium. Data represent the means of eight measurements. SE was less than 2% for each mean.

Because earlier work showed that Fe-EDTA was responsible for the photosensitized degradation of IAA in culture medium (3, 14), we conducted experiments to determine whether the photochemical activity of Fe-EDTA was related to the light-induced growth reduction. Although root growth was reduced when Fe-EDTA was omitted from the medium, there was no light-dependent growth reduction as seen in Murashige and Skoog medium containing Fe-EDTA (Table I). Furthermore, like the photosensitized degradation of IAA by Fe-EDTA (14), removal of UV and blue light with a yellow filter prevented the complete medium from becoming inhibitory (Table I). Thus, Fe-EDTA appears to be the source of the photochemistry responsible for the light-induced growth inhibition.

Because flavin-catalyzed photooxidation of EDTA leads to formaldehyde production (6), we investigated the possibility that formaldehyde is formed in light-exposed nutrient medium. GC-MS analysis of 2,4-DNPH-derivatized liquid medium that had been incubated for 13 d under 200 μmol m^{-2} s^{-1} of white light showed the presence of characteristic ions for formaldehyde-2,4-DNPH at m/e 210 (m^+), 180, 122, 79, 63, and 51 (7). Formaldehyde-2,4-DNPH was not detected in medium incubated in the dark or under yellow-filtered light.

[1] Abbreviation: 2,4-DNPH, 2,4-dinitrophenylhydrazone.

Table I. *The Effect of Fe-EDTA on Light-Dependent Reduction of Root Growth in Nutrient Culture Medium*

Complete culture medium and medium lacking Fe-EDTA were incubated at 23°C for 5 d in the dark and under unfiltered and yellow-filtered light at 200 μmol m^{-2} s^{-1}. Three-day-old seedlings were then placed on the preincubated media and their root growth was measured after incubation in the dark for 2 d. Data represent the mean of 20 measurements ± SE.

	Light Treatment		
	Dark	Unfiltered	Filtered
		mm	
+Fe-EDTA	13.4 ± 0.6	6.6 ± 0.5	12.6 ± 0.4
−Fe-EDTA	6.5 ± 0.4	6.1 ± 0.4	6.7 ± 0.3

Formaldehyde and glyoxylic acid were quantified by HPLC of 2,4-DNPH-derivatized liquid medium that had been incubated under various light conditions (Fig. 2). Formaldehyde reached 110 μM in medium incubated in white light. The yellow filter, which blocks formation of the light-induced inhibition (Table I) and degradation of IAA (14), kept the formaldehyde level below 10 μM. In the dark-incubated medium, the level of formaldehyde also remained less than 10 μM (data not shown). Glyoxylic acid increased slightly in white light-incubated medium but not under the yellow filter (Fig. 2). However, glyoxylic acid levels were always less than 20% of the formaldehyde concentration in white light. Glyoxylic acid is a degradation product of EDTA that can spontaneously degrade into formaldehyde and CO_2 (4), and therefore would not be expected to accumulate to high levels.

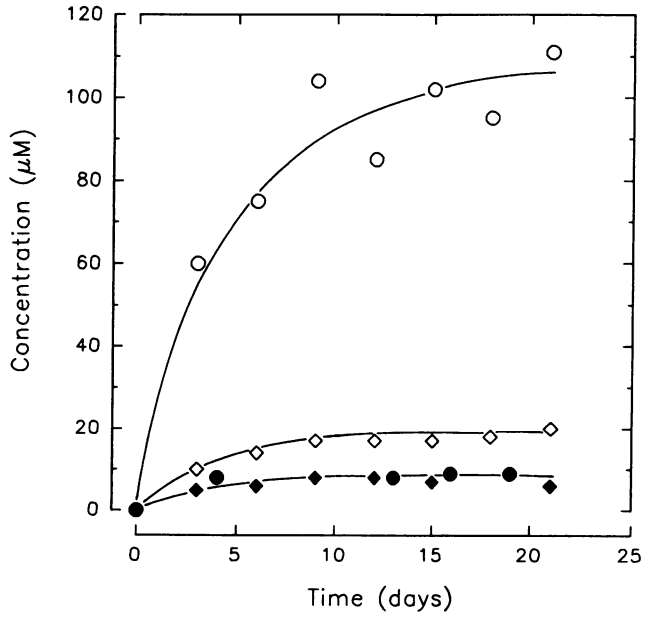

Figure 2. Time-course of light-induced formaldehyde and glyoxylic acid production in liquid culture medium. Open symbols are for white light and closed symbols are for yellow-filtered light. PPFD at the surface of the culture dishes was 200 μmol m^{-2} s^{-1} for both light treatments. Formaldehyde (○), glyoxylic acid (◇).

Because formaldehyde and glyoxylic acid are produced in light-exposed medium, their toxicity to root growth was determined (Fig. 3). *Arabidopsis* root growth was inhibited by both compounds. The concentration giving 50% inhibition was 200 μM for formaldehyde and 125 μM for glyoxylic acid. These values are higher than the concentrations found in medium that had been exposed to sufficient white light to cause complete inhibition of *Arabidopsis* root growth (Fig. 2). For example, the level of formaldehyde in the medium after 15 d exposure to 200 μmol m^{-2} s^{-1} of white light was about 100 μM, enough to inhibit root growth by only 25%. In the same medium, the amount of glyoxylic acid produced was enough to cause less than 5% inhibition. Thus, formaldehyde and glyoxylic acid production by EDTA photooxidation can account for only part of the light-induced growth reduction.

The pH of Murashige and Skoog culture medium is 5.8 (12). At that pH, unchelated iron rapidly forms insoluble ferric oxides which are unavailable to plant tissues, while the concentrations of Fe^{2+} and Fe^{3+} are too low to be of significance to plant cells (9). Thus, EDTA and iron are added together to the medium to keep the iron soluble (12). The photooxidative degradation of EDTA documented above would reduce the level of chelated iron in the culture medium and the unchelated iron would precipitate as unavailable ferric oxides (9). Because iron is essential for plant growth, the loss of available iron from the medium could lead to reduced growth.

The effect of iron availability on *Arabidopsis* root growth was tested by adding different amounts of chelated iron to

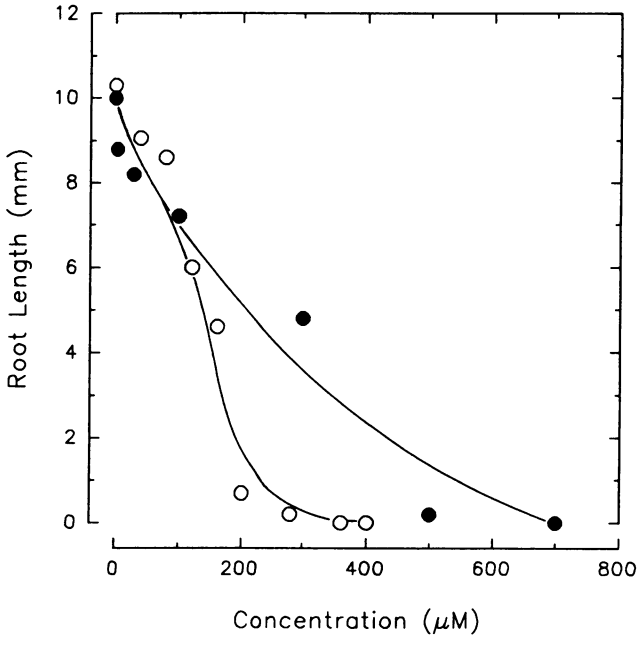

Figure 3. Inhibition of *Arabidopsis* root growth by formaldehyde and glyoxylic acid. Formaldehyde (○) and glyoxylic acid (●) were added to sterilized liquid culture medium after it cooled to 22°C. Three-day-old seedlings were then placed in the media and their root growth was measured after incubation in the dark for 2 d. Each data point represents the mean for 10 measurements. SE was less than 6% for each mean.

the medium either by varying the concentration of EDTA in medium containing 100 μM $FeSO_4$, or by changing the amount of both $FeSO_4$ and EDTA (Fig. 4). In both situations, as the concentration of chelated iron decreased there was a progressive decrease in root growth. In the absence of available iron, root growth was inhibited by about 60% compared with growth in complete medium. The extent of light-induced EDTA degradation was determined by measuring the amount of chelated iron remaining in solution after exposing solutions of Fe-EDTA to different light conditions (Fig. 5). The loss of chelated iron increased with increasing light intensity but was negligible in the dark for up to 13 d. Thus, iron-catalyzed photooxidation of EDTA can rapidly render Murashige and Skoog medium iron-deficient and can account for much of the light-induced growth reduction.

The combination of the loss of available iron and the production of toxic materials caused by Fe-catalyzed photooxidation of EDTA can account for most, but not all, of the growth reduction observed in light-exposed culture medium. The amine residue formed upon oxidation of EDTA (5) needs to be identified and tested for toxic effects. Other components of the medium, such as sucrose vitamin supplements, and redox-active ions may also participate in the complex photochemistry of plant tissue culture medium and contribute in some way to reduced growth in light-exposed Murashige and Skoog medium.

Replacing EDTA with other chelators, such as tartaric or citric acid, is not likely to eliminate the loss of available iron because several di- and tricarboxylic acid chelators have also been shown to be photooxidized by Fe^{3+} (1). Tertiary amines

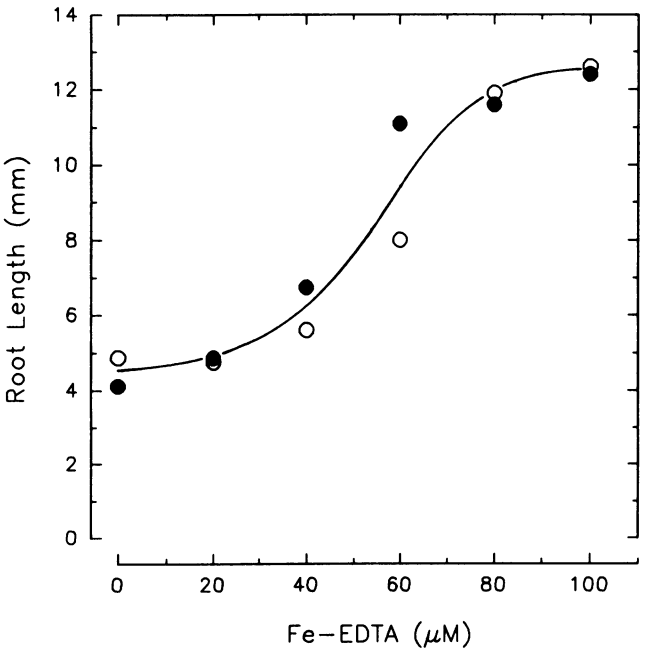

Figure 4. Effect of iron availability on *Arabidopsis* root growth. The available iron in liquid culture medium was varied by the addition of different amounts of Fe-EDTA (○), or by adding different concentrations of EDTA to medium containing 100 μM $FeSO_4$ (●). Data represent the mean of 10 measurements. SE was less than 4% for each mean.

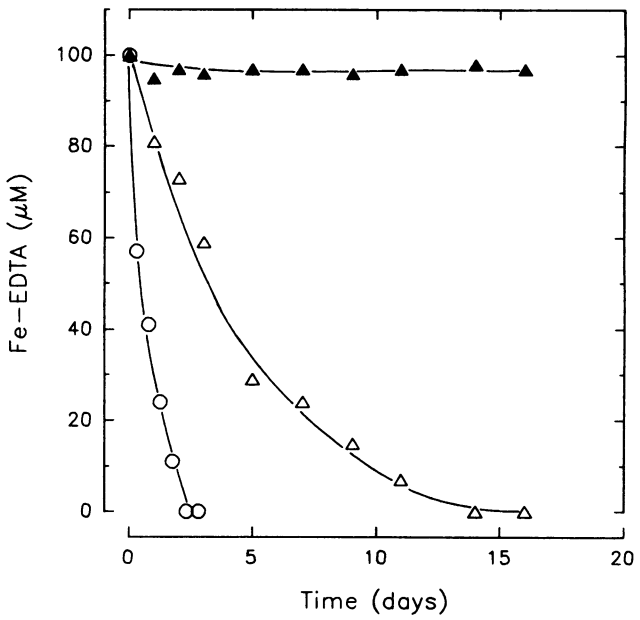

Figure 5. Effect of light on the loss of chelated iron. The amount of soluble chelated iron was measured in samples of Fe-EDTA (1 mM) that were incubated in 200 μmol m^{-2} s^{-1} (○) and 80 μmol m^{-2} s^{-1} (△) white light and in the dark (▲).

(like EDTA) are extremely susceptible to oxidation by photosensitized riboflavin (6), and presumably by Fe^{3+} as well, and therefore other tertiary amine chelators would probably not be much more stable than EDTA. Also, while it may be possible to prevent formaldehyde formation by using different chelators, the production of other potentially toxic photodegradation products must be considered. For example, photodegradation of Fe-citrate can lead to acetone production (1). Moreover, the Fe-photosensitized degradation of IAA will still occur because it does not depend strongly on the iron being chelated (3).

Fortunately, the use of light filters to remove UV and blue light, such as the yellow acrylic used here, provides a simple and effective means of eliminating the photochemical modifications of the medium that are associated with iron (14). Because the photochemistry of Fe-EDTA is driven most strongly by UV and blue light, precautions should be taken to prevent the media from being exposed to the light treatments when studying plant responses to UV or blue light in tissue cultures.

Although the growth experiments presented here were conducted with a simple root growth bioassay, it is reasonable to expect that at least some of the variability observed in plant tissue cultures may be the result of photochemical modifications of the media (14). The light-induced degradation of EDTA reported in this paper, and the degradation of IAA reported elsewhere (3, 14), are caused by iron-catalyzed photochemistry. In most plant tissue culture media formulations, Fe-EDTA is the iron source (2). Thus, the results presented in this paper for Murashige and Skoog medium will also apply to other media. Experiments are underway to investigate the impact of light-induced formaldehyde production, loss of

iron, and hormone degradation on the growth of tissue cultures of several plant species.

In summary, we have shown that Fe-EDTA is photochemically degraded in Murashige and Skoog medium. Photooxidation of EDTA results in formaldehyde formation, which is toxic to plant growth, and the loss of chelated iron, which renders the medium iron-deficient. The photodegradation of EDTA depends on light intensity and quality and correlates with a light-induced reduction in the growth-supporting capacity of tissue culture medium.

ACKNOWLEDGMENT

We thank Dr. Terry Graham for helping us with his HPLC system.

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